Exhibit A

MASSRY & GLASSOCK'S TEXTBOOK OF NEPHROLOGY

FOURTH EDITION

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LIPPINCOTT WILLIAMS & WILKINS
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Philadelphia • Baltimore • New York • London
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ratios are fast becoming a substitute for the 24-hour urine sample in the assessment of proteinuria. For the traditional method, the patient is instructed to collect all urine for a 24-hour period; usually the 24-hour collection period begins in the morning when the patient arises. The first voided specimen of the first morning is discarded, and the patient is asked to note the time. For the next 24 hours, all excreted urine is saved in the collection vessel, including any voided specimens occurring during the night. The patient is then instructed to awaken the following morning at the same time as the previous day, when the collection first began. The first voided specimen of the second morning should be collected and saved at the same time that the first voided specimen was discarded the day before. Ordinarily, quantitation of both the 24-hour protein and creatinine excretion is requested. In any given patient whose diet, renal function, and muscle mass remain constant, 24-hour urine creatinine excretion also remains approximately constant. Because of this, the latter serves as a measure of completeness of the 24-hour collection. The average man excretes 16 to 26 mg of creatinine per kilogram of ideal body weight per day; the average woman excretes 12 to 24 mg of creatinine per kilogram of ideal body weight per day. In aged individuals, urinary creatinine excretion may decline to 8 to 15 mg/kg of ideal body weight.

In patients suspected of having orthostatic proteinuria, 24-hour urine collections should be divided into two separate specimen bottles. The collection should proceed as described above, with the first morning specimen discarded. All subsequent urine collected during the course of the patient's daily activities should be collected in the first specimen container. After retiring for the evening, the patient should be instructed to remain in bed until the end of the collection period the next morning. Urine collected in the morning should be placed in the second specimen container and represents urine produced during recumbency. A comparison of supine and upright proteinuria can then be made. Patients with nocturia in whom this test is deemed necessary should be encouraged to void with the use of a bedpan or bedside urine collection container to avoid ambulation during the nighttime collection.

The timed 24-hour urine specimen is inconvenient to collect, and there are often difficulties in assessing the completeness of collection. For these reasons, some investigators have examined the utility of measuring the protein-to-creatinine ratio of random "spot" morning urine samples as a semiquantitative measure of proteinuria. This collection technique is rapidly becoming the standard collection technique in place of the 24-hour urine specimen in separating nephrotic-range from nonnephrotic-range proteinuria and also for following the clinical responsiveness of patients undergoing therapy for proteinuric states. A value greater than 3 mg protein/mg creatinine indicates nephrotic-range proteinuria. Available data have shown that the protein/creatinine ratio correlates with absolute and log-transformed 24-hour urine protein values ($P = .0001$ and $P < .0001$, respectively). The degree of proteinuria by this measure correlates with the rate of loss of renal function and is a risk factor for progression to end-stage renal disease.

The detection of small increases in urinary albumin excretion (more than 15 to 30 μg/min) in patients with insulin-dependent diabetes (incipient diabetic nephropathy) suggests the presence of early diabetic kidney disease and is highly predictive of the development of subsequent overt diabetic nephropathy. However, nonspecific factors may also induce small increments in proteinuria that might confound the prognostic usefulness of this test. Therefore, to maximize the prognostic accuracy of microalbuminuria, the following recommendations regarding urine collection have been suggested. Timed, overnight urine collections (preferably two or three) should be obtained. An overnight collection minimizes the contribution of an erect posture to albumin excretion. The patient should have adequate glycemic and blood pressure control during the collection, should avoid exercise, and the urine should be sterile. The patient should have no condition, other than diabetes, which may cause proteinuria. Urinary albumin is then usually measured by radial immunodiffusion, radioimmunoassay, or enzyme-linked immunosorbent assay (ELISA). Under these carefully controlled conditions, the presence of microalbuminuria identifies patients with insulin-dependent diabetes who are at high risk for developing overt diabetic nephropathy. In patients with adult-onset diabetes mellitus, the detection of microalbuminuria correlates better with cardiovascular and cerebrovascular disease than it does with nephropathy. Microalbuminuria may now also be detected using commercially available dipsticks which are more sensitive than those utilized for the standard urinalysis.

URINARY PROTEIN MEASUREMENT

Semiquantitative Measures of Proteinuria

Ideally, screening tests for proteinuria should be sensitive enough to identify low levels of abnormal proteinuria, but not so sensitive that normal low levels of proteinuria are detected and erroneously interpreted to be pathologic. The most commonly used semiquantitative test for proteinuria is the standard dipstick method, with a sensitivity of 10 to 30 mg/dl for albumin. The protein dipstick is a reagent stick impregnated with a color indicator, usually bromphenol blue, which is yellow at pH 3. Between pH 5 and 7, the indicator binds albumin and the color changes in proportion to the concentration of albumin in the urine. The dipstick is much less sensitive for detection of globulins, including low-molecular-weight proteins and light chains and may thus be falsely negative in the presence of tubular proteinuria, multiple myeloma, and overflow proteinuria because of the predominance of nonalbumin proteins in these conditions. Translation of the results of the dipstick method as an indicator of the amount of protein excreted requires knowledge of urine volume: 1+ proteinuria corresponds to 30 mg/dl protein and 4+ proteinuria indicates greater than 300 mg/dl. The dipstick proteinuria may also be falsely negative in the presence of very dilute urine. The dipstick method may falsely indicate abnormal proteinuria in the presence of a highly concentrated urine, in urine with a pH higher than 8, in the presence of phenazopyridine, or in urine contaminated with antiseptics such as chlorhexidine or benzalkonium (Table 101.3). An alternative

TABLE 101.3 Semiquantitative Studies for Proteinuria—False-Positive and False-Negative Results

	Method	
	Dipstick	Turbidometric
Concentrated urine	False +	False +
Dilute urine	False −	False −
Urine pH > 8	False +	False −
Contamination with antiseptics	False +	No effect
Tolbutamide metabolites	No effect	False +
Penicillins, cephalosporins (massive doses)	No effect	False +
Sulfisoxazole metabolites	No effect	False +
Phenazopyridine	False +	No effect
Radiographic contrast media	No effect	False +

means of assessing proteinuria semiquantitatively depends on protein precipitation by sulfosalicylic, trichloroacetic, or nitric acids or by heat and acetic acid. Turbidity is measured on a semiquantitative scale. These latter methods tend to be much more sensitive than the dipstick method for detecting proteinuria as low as 3 to 5 mg/dL. False-positive results are more common. Such false-positive results occur when testing highly concentrated urines with radiocontrast agents, sulfonamide and tolbutamide metabolites, and high concentrations of penicillins or cephalosporins. False-negative results may be seen in the presence of highly buffered alkaline urine or highly dilute urine. Despite their relative lack of specificity, one advantage of the turbidometric measures is that they are more sensitive than the dipstick method to nonalbumin proteins in the urine, including globulins and glycoproteins. Therefore a discrepancy between the dipstick method and the turbidometric methods suggests the presence of proteinuria characterized primarily by nonalbumin proteins. The finding of significant proteinuria by precipitation methods not confirmed by dipstick would require further studies to rule out the presence of multiple myeloma or other lesions leading to tubular proteinuria.

Quantitative Measures of Proteinuria

The most common procedures for quantitating proteinuria in clinical laboratories in the United States are the turbidometric methods, with quantitation by a photometer or nephelometer. Proteinuria may also be quantitated by ultraviolet spectrophotometry at 210 nm, the wavelength at which albumin and globulin display similar absorbance. Ultraviolet spectrophotometry offers enhanced precision of measurement compared with protein precipitation methods, but it is less sensitive and more time-consuming. The biuret method of measuring proteinuria depends on a color change occurring in the presence of alkaline copper sulfate and the biuret compound, quantitated spectrophotometrically at 557 nm. The biuret assay is more sensitive but more labor intensive than turbidometric methods. The micro-Kjeldahl method for measurement of protein nitrogen is the most accurate; however, it is expensive, tedious, and time-consuming and is therefore reserved for research purposes.

Qualitative Measures of Proteinuria

The qualitative aspects of abnormal proteinuria can be studied by the identification of specific proteins or classes of proteins using a variety of methods, including electrophoresis on cellulose acetate or polyacrylamide, immunoelectrophoresis in agarose gel, radial or double immunodiffusion, radioimmunoassay, and ELISA. Many of these methods may be readily adapted to provide quantitative information (e.g., densitometric scanning). Such techniques are particularly helpful for characterizing the pathogenetic mechanisms for abnormal proteinuria. The identification of a monoclonal paraprotein in urine would suggest overflow proteinuria, such as in multiple myeloma or primary amyloidosis. The presence of a high β_2-microglobulin excretion relative to albumin excretion would indicate a tubular origin of the proteinuria. The specific detection of small quantities of albumin (microalbuminuria) may be useful in the evaluation of early stages of diabetic nephropathy. In addition, microalbuminuria appears to be a general risk factor for cardiovascular and cerebrovascular mortality and morbidity, not just in diabetics, but also in the general population. This may suggest the utility of microalbuminuria as a marker for generalized endothelial dysfunction.

The specific immunologic measurements of fibrinogen or fibrin degradation products (fibrinopeptide A), complement proteins (C3 or C4), or tubular glycoproteins may be of clinical value in specific disorders such as crescentic glomerulonephritis (elevated excretion of fibrin degradation products), Alport's syndrome (elevated excretion of a fragment of C3), or acute tubular necrosis (elevated excretion of proximal renal tubular brush border antigen). The presence of the terminal component of complement [membrane attack complex (MAC)] in the urine may correlate with disease activity in membranous nephropathy.

A rapidly growing literature also demonstrates the presence of growth factors, cytokines, and extracellular matrix and albumin fragments in the urine of patients with proteinuria, and numerous studies have been performed to demonstrate that these products may be toxic to the tubulointerstitium. The interaction of these proteins with tubular cells are thought to mediate, at least in part, the tendency for progressive renal dysfunction in patients with proteinuria. Finally, experimental evidence suggests that specific proteins may be found in urine as markers of genitourinary malignancy.

PROTEIN SELECTIVITY

Calculation of a ratio of urinary clearances of several proteins, often referred to as *protein selectivity*, has been used to assess the degree of damage to the glomerular permeability barrier (Chapter 4). This can be accomplished by simultaneous measurement of the serum and urine concentrations of several proteins of differing molecular size (e.g., IgG and transferrin). The selectivity index is then calculated as $(U_{IgG}/P_{IgG}) \div (U_{transferrin}/P_{transferrin})$, where U and P represent the concentration of protein in urine and plasma, respectively. Values of less than 0.1 for this ratio indicate highly selective proteinuria and thereby less severe glomerular damage.

Fractional IgG clearance has also been used to indicate the extent of disruption of the glomerular permselectivity barrier (see Chapter 4). This is determined by measuring the urine and plasma concentrations of IgG and inulin. Fractional IgG clearance is then calculated as $(U_{IgG}/P_{IgG}) \div (U_{inulin}/P_{inulin})$. Values of less than 0.001 for this ratio indicate highly selective proteinuria.

Selected Readings

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